

# Changes in Activities of Enzymes of Carbon Metabolism in Leaves during Exposure of Plants to Low Temperature<sup>1</sup>

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## ABSTRACT

The aim of this study was to determine the response of photosynthetic carbon metabolism in spinach and bean to low temperature. (a) Exposure of warm-grown spinach and bean plants to 10°C for 10 days resulted in increases in the total activities of a number of enzymes, including ribulose 1,5-bisphosphate carboxylase (Rubisco), stromal fructose 1,6 bisphosphatase (Fru 1,6-P<sub>2</sub>ase), sedoheptulose 1,7-bisphosphatase (Sed 1,7-P<sub>2</sub>ase), and the cytosolic Fru 1,6-P<sub>2</sub>ase. In spinach, but not bean, there was an increase in the total activity of sucrose-phosphate synthase. (b) The CO<sub>2</sub>-saturated rates of photosynthesis for the cold-acclimated spinach plants were 68% greater at 10°C than those for warm-acclimated plants, whereas in bean, rates of photosynthesis at 10°C were very low after exposure to low temperature. (c) When spinach leaf discs were transferred from 27 to 10°C, the stromal Fru 1,6-P<sub>2</sub>ase and NADP-malate dehydrogenase were almost fully activated within 8 minutes, and Rubisco reached 90% of full activation within 15 minutes of transfer. An initial restriction of Calvin cycle fluxes was evident as an increase in the amounts of ribulose 1,5-bisphosphate, glycerate-3-phosphate, Fru 1,6-P<sub>2</sub>, and Sed 1,7-P<sub>2</sub>. In bean, activation of stromal Fru 1,6-P<sub>2</sub>ase was weak, whereas the activation state of Rubisco decreased during the first few minutes after transfer to low temperature. However, NADP-malate dehydrogenase became almost fully activated, showing that no loss of the capacity for reductive activation occurred. (d) Temperature compensation in spinach evidently involves increases in the capacities of a range of enzymes, achieved in the short term by an increase in activation state, whereas long-term acclimation is achieved by an increase in the maximum activities of enzymes. The inability of bean to activate fully certain Calvin cycle enzymes and sucrose-phosphate synthase, or to increase nonphotochemical quenching of chlorophyll fluorescence at 10°C, may be factors contributing to its poor performance at low temperature.

complete in evergreen woody species that are subject to large seasonal variations in temperature, such as *Eucalyptus* species and the desert evergreen, *Nerium oleander*. For such plants acclimated to low temperature, temperature response curves for photosynthesis indicate an increased photosynthetic capacity over a wide range of temperatures (2, 7). The increases in photosynthetic capacity that result from acclimation to a lower growth temperature could be the result of a number of factors, as plants acclimating to low temperature show increases in, for example, soluble protein, the rate of electron transport, and in the activities of enzymes such as Rubisco and the stromal Fru 1,6-P<sub>2</sub>ase,<sup>2</sup> which parallel the increase in photosynthetic capacity (2, 3).

There are a number of other reports of increases in Rubisco at lower temperatures, for example, in the arctic-alpine species *Oxyria digyna* (5), in the C<sub>4</sub> plant *Atriplex lentiformis* (24), and in the grass *Dactylis glomerata* (30). Gas-exchange studies also support the view that Rubisco increases after growth at lower temperatures. Ferrar *et al.* (8) estimated Rubisco activity from the initial slope of the response of the assimilation rate to the intercellular concentration of CO<sub>2</sub>. They found that *N. oleander* and a range of *Eucalyptus* species acclimated to low temperature exhibit a higher initial slope than that in warm-acclimated plants, indicating parallel changes in Rubisco activity *in vivo* and photosynthetic capacity in plants acclimated to low temperature. There is evidence of concomitant increases in stromal Fru 1,6-P<sub>2</sub>ase activity in plants of *N. oleander* transferred to a lower temperature (2). Gas-exchange analysis of *Eucalyptus* species also revealed a general increase in RuBP-regeneration capacity under such conditions (7). However, in all of these studies there is a lack of information about the effect of temperature on the activation states of Rubisco and of other light-activated enzymes of the Calvin cycle, or of sucrose synthesis, the capacity of which can markedly affect CO<sub>2</sub> assimilation at low temperatures (16).

There is abundant evidence for photosynthetic acclimation to temperature in a wide range of plants (2, 3, 8, 16, 21, 23, 24). Acclimation to a shift in temperature is evidenced by altered temperature optima and by increases in photosynthetic rates at the growth temperature. Acclimation is most

<sup>2</sup> Abbreviations: Fru 1,6-P<sub>2</sub>, fructose 1,6-bisphosphate; Fru 6-P, fructose 6-P; Glc 6-P, glucose 6-P; NADP-MDH, NADP<sup>+</sup>-malate dehydrogenase; PEP, phosphoenolpyruvate; Glc-P isomerase, phosphoglucose isomerase; RuBP, ribulose 1,5-bisphosphate; Sed 1,7-P<sub>2</sub>, sedoheptulose 1,7-bisphosphate; SPS, sucrose-P synthase; MDH, malate dehydrogenase; q<sub>p</sub>, photochemical quenching; q<sub>n</sub>, nonphotochemical quenching; Φ<sub>PSII</sub>, quantum efficiency of photosynthetic electron transport by PSII; Q<sub>10</sub>, ratio of reaction velocities at t°C and at t + 10°C; F<sub>o</sub>, fluorescence level when all PSII centers are open; F<sub>m</sub>, fluorescence yield when all centers are closed.

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In addition to its long-term effects on photosynthetic capacity, temperature can influence the rate of photosynthetic carbon assimilation in the short term in very different ways. In high light and high CO<sub>2</sub>, photosynthesis shows a very steep response to temperature, whereas at very low PPFD or low CO<sub>2</sub>, photosynthesis shows a flatter response to temperature (2, 3, 15) (*i.e.* the  $Q_{10}$  is close to unity). Thus, there will be situations in which the Calvin cycle, for example, will be required to operate at the same rate regardless of changes in temperature. This requires the operation of temperature-compensating mechanisms that act to increase enzyme activity at lower temperatures (11), which could conceivably be achieved by light activation in the case of Calvin cycle enzymes.

We have studied the response of leaves of spinach and bean plants to low temperature (10°C) by monitoring changes in the short term (min) and in the long term (over 10 d) in the total activity and, when appropriate, the *in vivo* activation state of some enzymes of the Calvin cycle, sucrose synthesis, and glycolysis. In short-term experiments, we have determined the changes in the activation state of Rubisco, stromal Fru 1,6-P<sub>2</sub>ase, and NADP-MDH, as well as changes in the amounts of metabolites associated with the Calvin cycle and changes in Chl fluorescence quenching when leaf discs are exposed to 10°C. We show that, in addition to the need for long-term increases in the activities of various enzymes, a rapid activation or maintenance of activation of critical enzymes is needed to increase photosynthetic capacity at low temperatures.

## MATERIALS AND METHODS

### Growth of Plant Material

Seeds of spinach (*Spinacia oleracea* L. cv Sobito) and bean (*Phaseolus vulgaris* L. cv Dwarf) were germinated in a greenhouse. Young seedlings (about 6 weeks old) with at least three fully expanded, large leaves were transferred to 1.3 L pots containing composted medium with a high nutrient content. The seedlings were then placed in a growth cabinet with a 10-h photoperiod set at 24°C (light) and 20°C (dark). The PPFD at the height of the plants was 300  $\mu\text{mol quanta}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ . After 7 d, the plants had acclimated fully to these growth conditions. In spinach, second and third leaves were used that had reached full expansion by day 3 of the warm-acclimation period. In bean, the leaves were fully expanded 1 d before the plants were placed into the warm growth conditions. The plants were fertilized with a complete nutrient solution on day 5 of the warm-acclimation period and on days 2 and 8 of the long-term, cold-acclimation experiments. For the long-term exposure to 10°C, the plants were first sampled from the 24°C cabinet to determine enzyme activity or photosynthesis rate 4 h into the light period. They were then transferred to a cabinet with the same photoperiod and PPFD but with a 10°C (light) and 7°C (dark) regimen.

### Experimental Procedure

Leaf discs (1.5 cm<sup>2</sup>) were used for all the analyses, and all discs were removed from the same leaf. In long-term experiments, the leaf discs were cut from plants within the growth cabinet and immediately plunged into liquid N<sub>2</sub> for storage.

For short-term experiments, several leaf discs were removed from the leaf and placed on moist filter paper in the light at a PPFD of 240  $\mu\text{mol quanta}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  under a stream of air. Spinach discs were placed with the adaxial surface facing up, but the bean discs were placed with their abaxial surface facing upward because of the low number of stomata on the upper epidermis. The leaf discs were allowed to equilibrate at a temperature of 27°C for 50 min. After this equilibration period, discs were immediately transferred at the same PPFD to moist filter paper cooled to 8°C. The temperature of similar leaf discs, measured using a thermocouple, dropped to 12°C during the 15 s after transfer and reached a constant  $10 \pm 0.5^\circ\text{C}$  within 35 s. At the appropriate time, the discs were plunged into N<sub>2</sub>. For the short-term experiments, one disc was extracted for each enzyme assayed, and five discs were combined for metabolite determination.

### Gas-Exchange Measurements

CO<sub>2</sub>-saturated rates of O<sub>2</sub> evolution from leaf discs (2.5 cm<sup>2</sup>) were measured in a leaf-disc O<sub>2</sub> electrode (Hansatech Ltd., King's Lynn, UK).

### Enzyme Activities

The frozen leaf discs were ground to a fine powder at the temperature of liquid N<sub>2</sub> in a mortar and pestle. The powder was extracted in a glass-in-glass homogenizer containing 1 mL ice-cold extraction medium that always contained 2% (w/v) PVP and 0.1% (v/v) Triton X-100. In addition, the medium for each enzyme extraction contained: Rubisco, 50 mM Tris-HCl (pH 8.0), 10 mM MgCl<sub>2</sub>, 1 mM EDTA, 15 mM 2-mercaptoethanol; stromal Fru 1,6-P<sub>2</sub>ase, the same as for Rubisco, but with the addition of 1 mM Fru 1,6-P<sub>2</sub>; Sed 1,7-P<sub>2</sub>ase, 50 mM Tris-HCl (pH 8.2), 10 mM MgCl<sub>2</sub>, 1 mM EDTA, 20 mM DTT; cytosolic Fru 1,6-P<sub>2</sub>ase and PEP carboxylase, 50 mM Hepes (pH 7.0), 5 mM MgCl<sub>2</sub>, 1 mM EDTA, 15 mM 2-mercaptoethanol; SPS, 50 mM Hepes (pH 7.4), 5 mM MgCl<sub>2</sub>, 1 mM EDTA, 10% (v/v) glycerol; pyruvate kinase, 50 mM Hepes (pH 7.0), 2 mM MgCl<sub>2</sub>, 1 mM EDTA, 5 mM DTT; Glc-P isomerase and hexokinase, 50 mM Tris-HCl (pH 8.1), 5 mM MgCl<sub>2</sub>, 10 mM 2-mercaptoethanol; NADP-MDH, 50 mM Tris-HCl (pH 8.2), 1 mM EDTA, 15 mM 2-mercaptoethanol.

An aliquot of the whole leaf extract was taken to determine Chl content (1), and the remainder was centrifuged for 10 s in an Eppendorf microfuge. The entire procedure took less than 1.25 min from homogenization to assay. All enzymes except Sed 1,7-P<sub>2</sub>ase and SPS were assayed spectrophotometrically at 30°C. The assays were as follows: Rubisco, as in ref. 13 with 25  $\mu\text{L}$  extract; stromal Fru 1,6-P<sub>2</sub>ase, for initial activity the solution contained 50 mM Tris-HCl (pH 8.0), 10 mM MgCl<sub>2</sub>, 1 mM EDTA, 0.5 mM NADP<sup>+</sup>, 0.1 mM Fru 1,6-P<sub>2</sub>, 10 units each of Glc-P isomerase and Glc 6-P dehydrogenase, 25  $\mu\text{L}$  extract. For total activity, the solution was the same except that 4 mM Fru 1,6-P<sub>2</sub> and 10 mM DTT were included; Sed 1,7-P<sub>2</sub>ase, 50 mM Tris-HCl (pH 8.2), 10 mM MgCl<sub>2</sub>, 1 mM EDTA, 0.3 mM Sed 1,7-P<sub>2</sub>, 10 mM DTT, 100  $\mu\text{L}$  extract. The Sed 1,7-P<sub>2</sub>ase assays were terminated after 30 s by the addition of 0.4 mL 1 M HClO<sub>4</sub>, and the amount of Pi released by the reaction was determined as described by Leegood (17). Cyto-

soluble Fru 1,6-P<sub>2</sub>ase, 50 mM Hepes (pH 7.0), 2 mM MgCl<sub>2</sub>, 0.1 mM Fru 1,6-P<sub>2</sub>, 0.5 mM NADP<sup>+</sup>, 10 units each of Glc-P isomerase and Glc 6-P dehydrogenase, 100  $\mu$ L extract; pyruvate kinase, as described by Plaxton (25); PEP carboxylase, 50 mM Hepes (pH 7.0), 10 mM MgCl<sub>2</sub>, 5 mM PEP, 10 mM NaHCO<sub>3</sub>, 10 mM DTT, 0.15 mM NADH, 10 units malate dehydrogenase, 100  $\mu$ L extract; Glc-P isomerase, 50 mM Tris-HCl (pH 8.2), 5 mM MgCl<sub>2</sub>, 0.2 mM NAD<sup>+</sup>, 2 mM Fru 6-P, and 3 units Glc 6-P dehydrogenase; hexokinase, 50 mM Tris-HCl (pH 8.0), 5 mM MgCl<sub>2</sub>, 0.2 mM NAD<sup>+</sup>, 10 mM glucose, 1 mM ATP, and 3 units Glc 6-P dehydrogenase; NADP-MDH, for determining the initial activity, 50 mM Tris-HCl (pH 8.2), 1 mM EDTA, 2 mM oxaloacetic acid, 0.15 mM NADPH, 100  $\mu$ L extract. For the total activity, 20 mM DTT was included. To activate NADP-MDH fully *in vitro*, 30 mM DTT was added to the extract and incubated for 30 min at room temperature under an atmosphere of N<sub>2</sub>. SPS, as described by Stitt *et al.* (29) for full activity, and under limiting conditions with 5 mM Pi, except that 5% (v/v) glycerol was included and 100  $\mu$ L extract was used. SPS assays were terminated after 15 min by boiling. Controls without Fru 6-P and Glc 6-P were included. After centrifugation of the boiled assay solutions, the amount of UDP formed was determined spectrophotometrically (29) in 50 mM Hepes (pH 7.0), 5 mM MgCl<sub>2</sub>, 2.5 mM PEP, 0.1 mM NADH, 10 units each of lactate dehydrogenase and pyruvate kinase, using 100  $\mu$ L SPS assay solution. The amount of UDP standard recovered in the presence and absence of Pi with plant extract was determined using the SPS assay solution but in the absence of Fru 6-P, Glc 6-P, and UDP-Glc. Essentially 100% of the UDP was recovered using spinach extracts and 72% was recovered using extracts of bean at 0 and 2 d at 10°C, but 51% was recovered using extract of bean leaves from plants exposed to 10°C for 10 d. The presence of Pi had no effect on the amount of UDP recovered in any of the plants.

The soluble protein content of the whole-leaf extracts was determined by the method of Bradford (4).

### Metabolite Analysis

Metabolites were extracted in 1 M HClO<sub>4</sub> from leaf discs frozen in liquid N<sub>2</sub> as described by Labate *et al.* (15). The amount of phaeophytin was determined by the method of Vernon (31). The amounts of RuBP and glycerate-3-P were measured according to Doncaster *et al.* (6). Triose-P were determined spectrophotometrically in an assay solution containing 50 mM Hepes (pH 7.5), 1 mM NaH<sub>2</sub>AsO<sub>4</sub>, 1 mM EDTA, 1 mM NAD<sup>+</sup>, and 10 units each of triose-P isomerase and glyceraldehyde-P dehydrogenase. The amounts of Fru 1,6-P<sub>2</sub> and Sed 1,7-P<sub>2</sub> were determined separately by first adding only glyceraldehyde-P dehydrogenase and omitting triose-P isomerase. After the reaction was complete, 5 units aldolase were added. This allowed measurement of the glyceraldehyde-P deriving from Fru 1,6-P<sub>2</sub>. Triose-P isomerase was then added to determine the amount of dihydroxyacetone-P deriving from both Fru 1,6-P<sub>2</sub> and Sed 1,7-P<sub>2</sub>. Hexose-P were measured as described by Lowry and Passonneau (19).

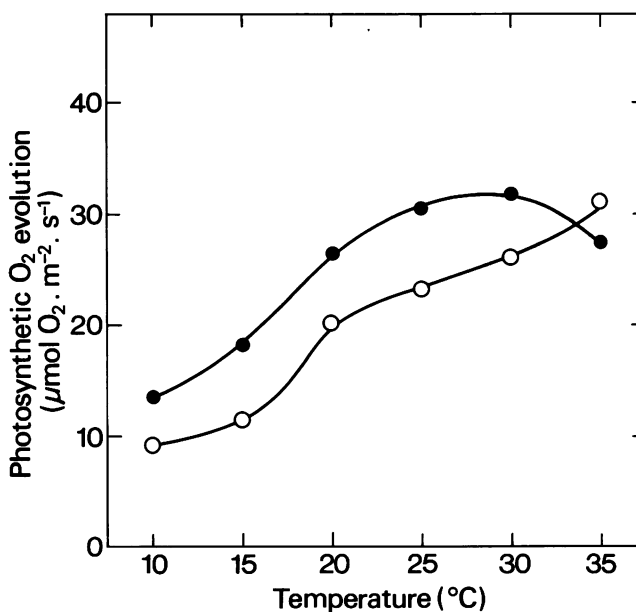
### Chl Fluorescence Analysis

A leaf disc was equilibrated on moist filter paper at 27°C and 240  $\mu$ mol quanta  $\cdot$  m<sup>-2</sup>  $\cdot$  s<sup>-1</sup>. Chl fluorescence was continuously monitored with a pulse modulation fluorometer (H. Walz, Effeltrich, FRG) after transfer to moist filter paper at 8°C and 240  $\mu$ mol quanta  $\cdot$  m<sup>-2</sup>  $\cdot$  s<sup>-1</sup>, as described above. This procedure meant that F<sub>0</sub> and F<sub>m</sub> could not be measured prior to the low-temperature treatment. To check for irreversible, or only slowly reversible, changes in q<sub>N</sub>, leaf discs were darkened (a) within a few seconds after transfer and were left darkened for at least 1 h to determine F<sub>0</sub> and F<sub>m</sub>, (b) after various periods of exposure to 10°C. F<sub>m</sub>/F<sub>0</sub> ratios were then determined and compared with those observed at 27°C or immediately after darkening the leaf at 10°C. These high values (approximately 5) were only obtained after short exposures to 10°C (5–10 min). After longer exposures to 10°C, q<sub>N</sub> relaxed both incompletely and very slowly. The experimental procedure, therefore, involved an exposure to 10°C for 280 s, during which no photoinhibition occurred. The disc was then dark adapted for 1 h before values of F<sub>0</sub> and F<sub>m</sub> were determined.  $\phi_{PSII}$  was calculated according to the method of Genty *et al.* (9).

## RESULTS

### Changes in Photosynthetic Capacity in Spinach

At nearly all temperatures, spinach plants acclimated to 10°C exhibited higher rates of CO<sub>2</sub>-saturated photosynthesis than those grown at 24°C (Fig. 1). Rates of photosynthesis at 10°C were increased by 68%, from 9.1 to 13.4  $\mu$ mol O<sub>2</sub>  $\cdot$  m<sup>-2</sup>  $\cdot$  s<sup>-1</sup>.



**Figure 1.** Temperature dependence of photosynthetic O<sub>2</sub> evolution by spinach leaf discs acclimated for 10 d to 24°C (○) or 10°C (●). The photosynthetic rates were measured under CO<sub>2</sub>-saturated conditions at a PPFD of 400  $\mu$ mol quanta  $\cdot$  m<sup>-2</sup>  $\cdot$  s<sup>-1</sup>. Data are means of between 3 and 25 samples. Standard errors were less than 10% of the means.

s<sup>-1</sup>, following 10 d acclimation at 10°C. It should be noted that this acclimation occurred in spinach leaves that were fully expanded before they were exposed to the low temperature, as in the case of the *Eucalyptus* species and *N. oleander* (2, 8).

Although enzyme data are presented for experiments over 10 d, some spinach plants were maintained at 10°C for 20 d, but their enzyme activities were similar to those in plants held at 10°C for 10 d (data not shown). No substantial change occurred in total (fully activated) activity or initial activity (extracted and assayed under nonactivating conditions) for any enzyme that we studied in control plants at 24°C during the 10 d experiment (Table I). Leaves used throughout the 10 d experiment were fully expanded before the experiment began. Enzyme activities expressed on a fresh weight basis (data not shown) changed in a manner similar to those for activities expressed on a Chl basis. Because no change in Chl content occurred in spinach (Table I) and only a slight decrease occurred in bean (Table II) throughout the 10 d exposure to 10°C (data not shown), all enzymic data have been presented on a Chl basis. The increase in soluble protein content in spinach leaves held at 10°C for 10 d ranged from 15 to 92% (data not shown), but controls at 24°C lost 13% of their initial soluble protein over the 10 d period.

Rapid assay methods were employed to determine the *in vivo* activation states of two Calvin cycle enzymes, Rubisco and the stromal Fru 1,6-P<sub>2</sub>ase. The total (CO<sub>2</sub>/Mg<sup>2+</sup>-activated) activity of Rubisco increased with time at 10°C for spinach (Table I). After 10 d, total Rubisco activity was 20% greater

than at day 0. However, a more striking change was the almost complete *in vivo* activation of the enzyme by 2 d and throughout the remainder of the 10 d period. For control plants at 24°C, the activation state of the enzyme remained between 67 and 77% (Table I).

The total, *in vitro*-activated activity of stromal Fru 1,6-P<sub>2</sub>ase in spinach increased by 67% over the 10 d exposure of 10°C (Table I). As with Rubisco, the most striking change occurred in the activation state of this light-activated enzyme. Within 2 d at 10°C, the enzyme was fully activated *in vivo*, and the activation state remained high, such that the initial activity at 10 d was twice that at day 0 (Table I). In the plants held at 24°C, the percentage of enzyme that was activated ranged from 65 to 83%. In the case of Sed 1,7-P<sub>2</sub>ase, its instability in leaf extracts without high substrate levels and DTT present precluded measurement of the initial activity. However, the total activity of spinach Sed 1,7-P<sub>2</sub>ase almost doubled in plants at 10°C after 4 d (Table I).

We also measured activities of the three enzymes associated with sucrose synthesis; SPS, the cytosolic Fru 1,6-P<sub>2</sub>ase, and Glc-P isomerase. Activities of these enzymes increased by between 58 and 87% (Table I). Sucrose-P synthase from spinach is sensitive to Pi under limiting substrate conditions, and this sensitivity diminishes as the enzyme is activated. Sensitivity to Pi can be employed as a measure of the *in vivo* activation state of the enzyme (29). The activation state of SPS in the plants at 10°C was rather variable throughout the experimental period with no clear trend emerging (Table I). This variability may reflect variability in the rate of CO<sub>2</sub>

**Table I.** Changes in the Activities and Activation States of Enzymes in Spinach Leaves during Acclimation to Low Temperature

Plants were grown at 24°C and then transferred to a temperature of 10°C for up to 10 d. The data were collected from five spinach plants that were subjected to low temperature and sampled individually, 4 h into the light period on each sampling day, throughout each experiment. The experiments were repeated three times. Three control spinach plants were also sampled. The activation state of enzymes *in vivo* is shown in parentheses, as a percentage of total activity.

Enzyme Activity	Days at 10°C					Control Plants (Days at 24°C)	
	0	2	4	6	10	0	10
$\mu\text{mol} \cdot \text{h}^{-1} \cdot \text{mg}^{-1} \text{ Chl}$							
Rubisco							
Initial	332 ± 25	492 ± 55	685 ± 54	575 ± 121	570 ± 39	365 ± 29	326 ± 5
Total	478 ± 54	500 ± 59	692 ± 45	578 ± 122	575 ± 35	546 ± 27	423 ± 75
	(70%)	(99%)	(99%)	(100%)	(99%)	(67%)	(77%)
Fru 1,6-P <sub>2</sub> ase (stromal)							
Initial	234 ± 30	378 ± 26	408 ± 33	441 ± 6	480 ± 44	274 ± 59	209 ± 44
Total	318 ± 8	379 ± 25	430 ± 42	488 ± 37	529 ± 31	329 ± 36	324 ± 9
	(74%)	(100%)	(95%)	(90%)	(91%)	(83%)	(65%)
Sed 1,7-P <sub>2</sub> ase	208 ± 33	324 ± 24	399 ± 16	403 <sup>a</sup>	378 ± 33	n.d. <sup>b</sup>	n.d.
Fru 1,6-P <sub>2</sub> ase (cytosolic)	74 ± 10	80 ± 13	107 ± 13	112 ± 18	118 ± 20	84 ± 5	78 ± 7
Sucrose-P synthase							
-Pi	34 ± 3	39 ± 4	48 ± 4	56 ± 7	57 ± 7	35 ± 1	35 ± 9
+Pi	14 ± 3	15 ± 4	17 ± 6	28 ± 3	19 ± 5	9 ± 2	11 ± 4
	(41%)	(40%)	(36%)	(51%)	(32%)	(27%)	(31%)
Glc-P isomerase	196 ± 6	218 ± 4	211 ± 4	335 ± 4	366 ± 4	n.d.	n.d.
Hexokinase	13 ± 2	16 ± 2	17 ± 2	22 ± 2	25 ± 2	n.d.	n.d.
Pyruvate kinase	107 ± 9	102 ± 9	136 ± 24	127 ± 18	152 ± 24	98 ± 4	103 ± 10
PEP carboxylase	59 ± 13	61 ± 9	73 ± 23	77 ± 23	68 ± 28	54 ± 6	57 ± 4
Chl (mg · m <sup>-2</sup> )	273 ± 47	279 ± 48	269 ± 33	261 ± 21	253 ± 25	330 ± 29	305 ± 53

<sup>a</sup> Mean of two determinations.

<sup>b</sup> Not determined.

**Table II.** *Changes in the Activities and Activation States of Enzymes in Bean Leaves during Acclimation to Low Temperature*

Plants were grown at 24°C and then transferred to a temperature of 10°C for up to 10 d. The data were collected from five bean plants that were subjected to low temperature and sampled individually, 4 h into the light period on each sampling day, throughout each experiment. The experiments were done three times. Three control bean plants were also sampled and exhibited no increases in enzyme activity (data not shown). The activation state of enzymes *in vivo* is shown in parentheses, as a percentage of total activity.

Enzyme	Days at 10°C		
	0	2	10
Rubisco			
Initial	361 ± 11	382 ± 57	540 ± 54
Total	368 ± 16	406 ± 25	694 ± 36
	(98%)	(94%)	(78%)
Fru 1,6-P <sub>2</sub> ase (stromal)			
Initial	180 ± 17	200 ± 32	218 ± 36
Total	307 ± 48	290 ± 36	422 ± 31
	(59%)	(69%)	(52%)
Sed 1,7-P <sub>2</sub> ase	333 ± 54	393 ± 43	353 ± 135
Fru 1,6-P <sub>2</sub> ase (cytosolic)	23 ± 7	30 ± 2	38 ± 2
Sucrose-P synthase			
−Pi	17 ± 3	18 ± 3	11 ± 4
+Pi	13 ± 2	12 ± 3	3 ± 1
	(81%)	(71%)	(26%)
Pyruvate kinase	29 ± 4	53 ± 2	85 ± 9
PEP carboxylase	65 ± 9	82 ± 14	134 ± 9
Chl (mg · m <sup>−2</sup> )	242 ± 23	225 ± 20	212 ± 17

assimilation at the time of sampling (4 h into the light period) (29). Nevertheless, the activity of the enzyme from 10 d plants in the presence of 5 mM Pi was greater than the activity of the enzyme from plants at day 0. Therefore, it is likely that an increase in the *in vivo* capacity of SPS occurs in the plants during cold acclimation. Indeed, the overall capacity to synthesize sucrose appears to increase in these plants relative to the capacity in control plants, because Glc-P isomerase and the cytosolic Fru 1,6-P<sub>2</sub>ase activities also increased (Table I) and amounts of hexose-P doubled in leaves (measured 4 h into the light period; data not shown).

The increase in the respiratory capacity of plants grown at low temperature is well documented (23). As we have noted above, the activity of Glc-P isomerase, which participates in glycolysis as well as in sucrose synthesis, was 87% higher in leaf extracts from plants acclimated to 10°C than in the extracts from plants grown at 24°C (Table I). The activities of the glycolytic enzymes, pyruvate kinase and hexokinase, also increased appreciably in spinach plants at 10°C over the 10 d period. However, the activity of PEP carboxylase did not change significantly in spinach with a 10d exposure to low temperature (Table I).

### Changes in Photosynthetic Capacity in Bean

Measurements of the CO<sub>2</sub>-saturated assimilation rates in bean grown at 24°C showed that they fell to low values at 10°C (data not shown). For example, the mean rate (of four samples) of O<sub>2</sub> evolution measured at 25°C was 12.2 μmol O<sub>2</sub> · m<sup>−2</sup> · s<sup>−1</sup> and measured at 10°C was 2.3 μmol O<sub>2</sub> · m<sup>−2</sup> · s<sup>−1</sup>.

After 10 d at 10°C, the assimilation rate had fallen to 0.7 μmol O<sub>2</sub> · m<sup>−2</sup> · s<sup>−1</sup> when measured at 10°C. Although plants had no visible symptoms of stress during this period, after 14 d at 10°C, bean plants were beginning to senesce.

Warm-grown bean plants exposed to 10°C for 10 d showed increases in the total activities of Rubisco (89%), stromal Fru 1,6-P<sub>2</sub>ase (38%), and Sed 1,7-P<sub>2</sub>ase (29%) (Table II), as in spinach. Although the total activity of Rubisco increased in bean leaves, the percentage of the enzyme that was activated *in vivo* declined from 98 to 78% during the 10d exposure to 10°C (Table II). In addition, although the percentage of stromal Fru 1,6-P<sub>2</sub>ase in the activated state *in vivo* increased by day 2 from 59 to 69%, by day 10 it had declined to 52% (Table II). Thus, the underlying increase in the *in vivo* Calvin cycle capacity is probably less than the total activity measurements would suggest.

The capacity for sucrose synthesis, as judged from the activities of the cytosolic Fru 1,6-P<sub>2</sub>ase and SPS, is not as substantial in bean as in spinach leaves, nor does it increase appreciably after 10 d at 10°C (Table II). Even though the cytosolic Fru 1,6-P<sub>2</sub>ase activity in bean increased by 64%, the extractable activity of SPS declined and was much less than that from the cold-acclimated spinach plants, even allowing for lower UDP recovery in the assay of SPS (see "Materials and Methods"), the activity of this enzyme does not increase with time at 10°C. Furthermore, the sensitivity of bean SPS to 5 mM Pi increases the longer the plants are exposed to 10°C, suggesting that the *in vivo* activity of the enzyme could be lower than indicated by the total activity in leaf extracts.

### Short-Term Metabolic Changes after Chilling Spinach Leaf Discs

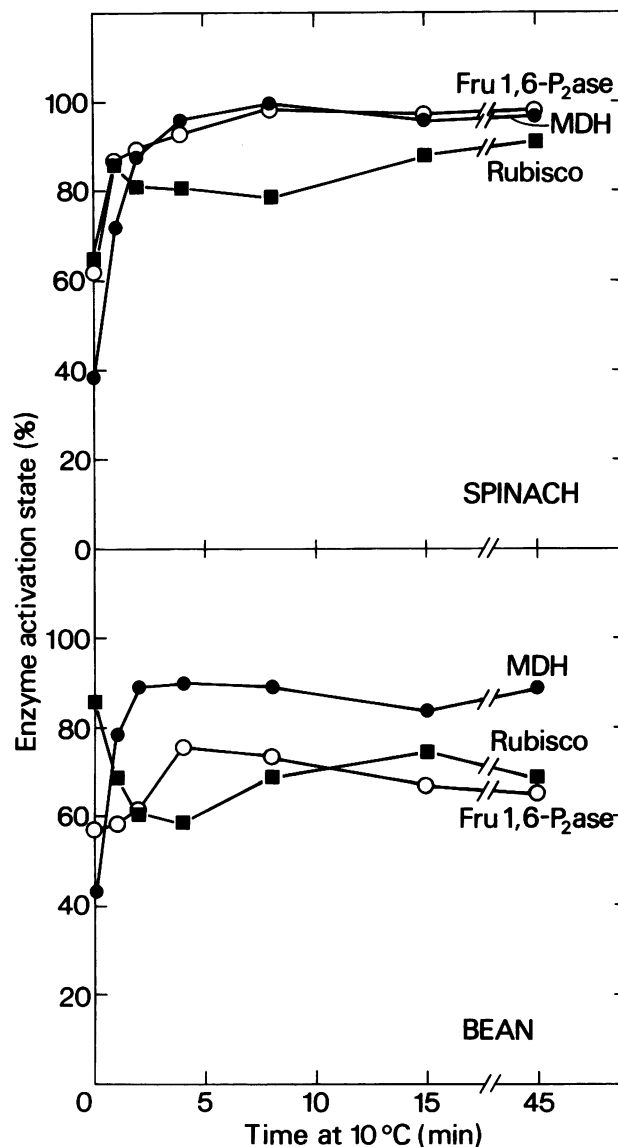
Leaf discs were rapidly transferred from a temperature of 27 to 10°C and samples taken at intervals thereafter. Within 1 min of the transfer of spinach leaf discs, the activation state of Rubisco increased from 65 to 86% (Fig. 2). After 45 min, the enzyme was 91% activated. Complete activation of the stromal Fru 1,6-P<sub>2</sub>ase and of NADP-MDH occurred within 8 min after transferring the discs to low temperature (Fig. 2).

Increases in several metabolites occurred after the spinach leaf discs were transferred to 10°C (Fig. 3). There was a transient increase in the amount of RuBP after 1 min, which would be consistent with a rapid reduction in carboxylation capacity upon transfer to 10°C. However, by 2 min the amount of RuBP had declined, probably reflecting activation of Rubisco (Fig. 2). There was an increase in glycerate-3-P, but the amount of triose-P remained essentially constant. Therefore, the ratio of triose-P/glycerate-3-P decreased, suggesting a restriction in the ability of the leaf to generate the ATP and/or NADPH needed to reduce glycerate-3-P to triose-P (18). The amounts of Fru 1,6-P<sub>2</sub> and Sed 1,7-P<sub>2</sub> increased (the latter by nearly eightfold). Although this increase indicates an initial restriction in the *in vivo* capacities of Fru 1,6-P<sub>2</sub>ase and Sed 1,7-P<sub>2</sub>ase after cooling, the ratio triose-P/RuBP, which is a measure of regeneration capacity within the Calvin cycle, decreased from 0.30 to 0.24 after 2 min at 10°C and remained near 0.28 thereafter, showing that enzyme activation was sufficient to remove any potential restrictions in Calvin cycle turnover at 10°C. The rise in these substrates is probably also a mechanism that maintains the flux through the bisphosphatases despite the temperature-dependent decreases in velocity. Hexose-P also increased, perhaps due to a restriction in the rate of sucrose synthesis (15).

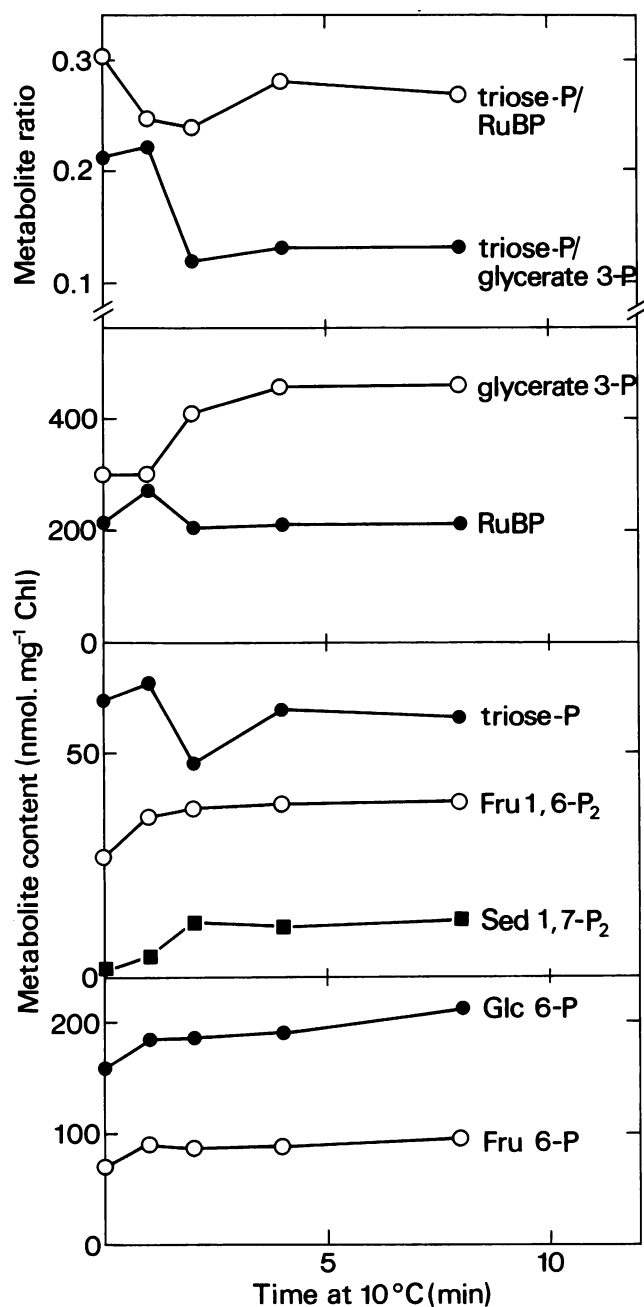
Chl fluorescence analysis in spinach leaf discs after transfer to 10°C (Fig. 4) revealed a transient decrease (at 100 s) in  $q_p$ , indicating transient reduction of the primary electron-accepting quinone of PSII,  $Q_A$ . There was also an initial decline in the quantum efficiency for  $\phi_{PSII}$  (9). These data are consistent with a fall and rise in the rate of photosynthesis, perhaps reflecting a lag period during metabolic readjustment at low temperature. There was also a continuous increase in  $q_N$ , which would be consistent with increased energization, due to a decrease in the utilization of the products of electron transport by photosynthesis and photorespiration, and to increased dissipation of excitation energy at low temperature.

### Short-Term Metabolic Changes after Chilling Bean Leaf Discs

In contrast with the short-term responses of the enzymes in spinach, the activation state of Rubisco *in vivo* increased slightly and then decreased after transfer of bean leaf discs from 27 to 10°C (Fig. 2), and the long-term trend was a deactivation of this enzyme over time after exposure to low temperature (Table II). The activation of stromal Fru 1,6-P<sub>2</sub>ase was also weak relative to that observed in spinach (Fig. 2). The activation state of the enzyme remained unchanged at about 58% for the first minute at 10°C, increased to 78% by 4 min, but declined again by 45 min. Despite the problems



**Figure 2.** Short-term changes in the activation states of NADP<sup>+</sup>MDH (●), Fru 1,6-P<sub>2</sub>ase (○), and Rubisco (■) after exposure to low temperature (10°C) in leaves of spinach and bean. Leaf discs were taken from warm-grown plants and illuminated at 27°C for 50 min. They were then transferred in the light to a temperature of 10°C. At each time point, leaf discs were rapidly plunged into liquid N<sub>2</sub> in the light, and enzymes assayed as described in "Materials and Methods." Data are means of three experiments. Standard errors were less than 10% of the means.



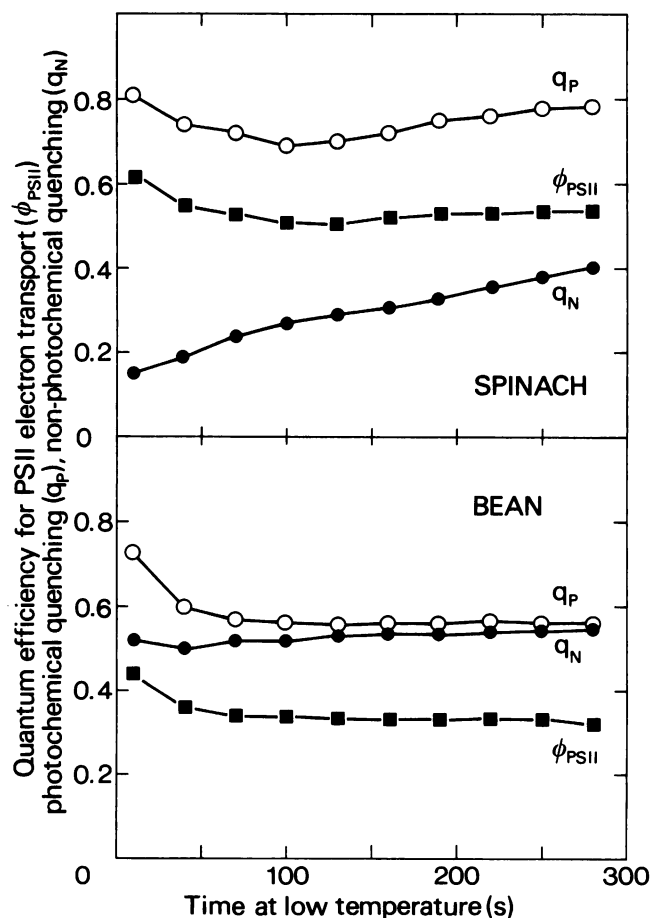
**Figure 3.** Short-term changes in metabolites and metabolite ratios after exposure to low temperature (10°C) in leaves of spinach. Leaf discs were taken from warm-grown plants and illuminated at 27°C for 50 min. They were then transferred in the light to a temperature of 10°C. At each time point, leaf discs were rapidly plunged into liquid N<sub>2</sub> in the light, and enzymes assayed as described in "Materials and Methods." Data are means of three experiments. Standard errors were less than 10% of the means.

in activating Rubisco and Fru 1,6-P<sub>2</sub>ase, the activation state of bean NADP<sup>+</sup>-MDH increased from 44 to 89% in 2 min and was maintained near that level for the duration of the experiment (Fig. 2).

Chl fluorescence analysis of bean leaf discs at 10°C showed that photochemical quenching and the quantum efficiency of PSII declined during the first minute (Fig. 4). Unlike spinach, there was no subsequent increase in  $q_p$  or in  $\phi_{PSII}$ , and non-photochemical fluorescence quenching in bean increased only slightly after exposure to low temperature, although in the longer term there was a steady decline in variable fluorescence that could be attributed to photoinhibition.

## DISCUSSION

Acclimation to low temperatures involves increases in photosynthetic capacity at the lower temperatures and must require an increase in the activity of any enzyme, such as



**Figure 4.** Short-term changes in photochemical ( $q_p$ ) (○) and nonphotochemical ( $q_N$ ) (●) Chl fluorescence quenching and in the quantum yield of photosystem II ( $\phi_{PSII}$ ) (■) after exposure of leaf discs of spinach and bean to low temperature (10°C). Leaf discs were taken from warm-grown plants and illuminated at 27°C for 50 min. They were then transferred in the light to a temperature of 10°C. Data are means of 10 separate experiments. Standard errors were less than 10% of the means.

Rubisco or Fru 1,6-P<sub>2</sub>ase, which might potentially limit the rate of photosynthesis at the lower temperature. To increase photosynthetic capacity, spinach increases the activities of a range of enzymes and, where possible, increases the degree to which these enzymes are activated (Table I, Fig. 2). (It should be noted that it is likely, but not certain, that activity increases measured at 30°C will be translated into capacity increases *in vivo* at 10°C, although this will depend upon changes in kinetic constants and metabolite contents.) As mentioned in the introduction, increases in the activity of Rubisco and Fru 1,6-P<sub>2</sub>ase at low temperature for several species have been reported. In experiments in which leaves of *N. oleander* were transferred from 45 to 20°C, the increase in the rate of CO<sub>2</sub> assimilation at lower temperatures, both during and after acclimation, correlated well with increases in the activities of Fru 1,6-P<sub>2</sub>ase and Rubisco, whereas activities of other enzymes (e.g. glycerate-3-P kinase, Glc-P isomerase, and phosphoglucomutase) increased, but to a lesser extent (2).

At least two factors will influence product synthesis after transfer to low temperature. First, carbohydrates will accumulate (and exercise a cryoprotective function) after transfer to low temperature as a consequence of decreased translocation (3). Second, the rate at which sucrose is synthesized can limit the rate of photosynthesis in the short term (16). Acclimation is likely to involve changes in the nature of stored carbohydrate, such as a switch to starch synthesis, increased export, and an increased capacity for sucrose synthesis that reflects the increase in photosynthetic capacity. In spinach, this increased capacity is provided by increases in the activities of the cytosolic Fru 1,6-P<sub>2</sub>ase, SPS, and Glc-P isomerase (Table I). Although the percentage of SPS activated *in vivo* did not change appreciably with acclimation to low temperature, the total activity did increase, so that the activity of the enzyme was higher in the cold-acclimated plants. However, the fact that the activation state of SPS was not increased by exposure to low temperature may be connected with the accumulation of carbohydrate at low temperature (data not shown). It should be noted that CO<sub>2</sub>-saturated photosynthetic rates in the spinach acclimated to low temperature were higher than those in plants grown at 24°C over almost the whole temperature range, including the higher growth temperature (Fig. 1). This behavior at high CO<sub>2</sub> concentrations is a feature that has also been observed in *N. oleander* (2), in *Larrea divaricata* (20), and in winter rye (12). Leaves from plants grown at higher temperatures cannot normally make full use of these high CO<sub>2</sub> concentrations because the capacity for sucrose synthesis limits photosynthesis, particularly at lower temperatures (16), whereas plants acclimated to the lower temperature have a higher intrinsic capacity for sucrose synthesis.

After exposure to cold, the content of a range of enzymes and of leaf soluble protein was increased. This agrees with a number of observations of increases in the amounts of a wide range of proteins at low temperature (23). The observed increases in Rubisco, and in soluble protein in general, have considerable implications for nitrogen use in plants at low temperature. They suggest that nitrogen-limited plants will be less able to acclimate to low temperature, in much the same way as nitrogen limits shade-sun acclimation (7). On the other hand, acclimation of photosynthesis to temperature in the

planktonic alga, *Skeletonema costatum*, results in rates of photosynthesis and respiration that are virtually the same at 8 and at 20°C, and is accompanied by a doubling of protein per cell. The price paid for this large increase in protein synthesis in *S. costatum* is a lower growth rate at the lower temperature (28).

In the short term, leaves require temperature-compensating mechanisms that act to increase enzyme activity at lower temperatures, because in low light or low CO<sub>2</sub>, Q<sub>10</sub> values for photosynthesis are near unity (2, 3, 15). This means that there will be situations in which some enzymic processes, such as RuBP carboxylation and Calvin cycle turnover, will be required to operate at the same rate over a range of temperatures. In principle, temperature compensation can be achieved by increasing the substrate affinity or increasing the amount of substrate at low temperature (11, 15). The data for spinach show that light activation of enzymes, such as Rubisco, the stromal Fru 1,6-P<sub>2</sub>ase, and NADP-MDH, is also acting as a temperature-compensating mechanism in leaves exposed to low temperature, thereby increasing the capacity of these steps. In the case of the bisphosphatases, capacity is likely to be further increased by an increase in the amounts of their substrates, although we must know more about the temperature-dependent properties of these enzymes before we can confidently predict what will happen to Calvin cycle fluxes at lower temperatures. Because the activation of Rubisco, stromal Fru 1,6-P<sub>2</sub>ase, and NADP-MDH is dependent on reducing power from electron transport, it is important to note that the almost complete activation of these enzymes in spinach occurs under conditions that reduce the efficiency of linear electron transport. A restriction in carbon assimilation, as would occur at low temperature, usually leads, through photosynthetic control, to an inactivation of redox-regulated enzymes (18). However, the decrease in the ratio triose-P/glycerate-3-P suggests that carbon assimilation is restricted relatively less than electron transport after transfer to low temperature.

Two previous studies also suggest that short-term activation of enzymes occurs at low temperature. Schnyder *et al.* (27) illuminated leaves of white clover for 60 min between 7 and 25°C. The initial (*in vivo*-activated) activity of Rubisco increased at low temperatures at all except the highest PPFDs. Kobza and Edwards (14) illuminated wheat leaves at temperatures ranging from 15 to 45°C and measured initial enzyme activities. Both Rubisco and Fru 1,6-P<sub>2</sub>ase showed higher extractable activities at the lower temperatures, implying that their activation states had increased.

Most studies of long-term changes in enzyme activity in chilling-sensitive plants have been made in rice. Maruyama *et al.* (20) showed that low temperature (15°C) largely prevented the normal developmental increases in Rubisco (perhaps from an inability to synthesize the small subunit [10]), the stromal Fru 1,6-P<sub>2</sub>ase, NADP-glyceraldehyde-P dehydrogenase, and a range of other enzymes, suggesting a specific curtailment of enzyme synthesis by chilling. In bean, which is also chilling-sensitive, the picture was rather different from that in rice or spinach. In bean, there was an ability to increase certain enzyme activities, but a limited capacity to activate enzymes. Although bean has the ability to increase the total activity of Rubisco and Fru 1,6-P<sub>2</sub>ase (Table II), this increase



did not result in an increase in photosynthetic capacity. Although samples from bean were taken in the middle of the photoperiod, we cannot exclude the possibility that the short-term inability to activate Rubisco or the long-term increases in Rubisco activity were due to changes in the tight-binding inhibitor, carboxyarabinitol-1-P (22), rather than to changes in activation state or to *de novo* synthesis. However, the high total activities for the enzyme in the short-term experiments would argue against the involvement of the inhibitor. On the other hand, if the doubling of Rubisco activity in bean represents enzyme synthesis, then it may be a means of attempting to overcome the inability of bean leaves to activate the enzyme.

In bean, it appears likely that the assimilation rate at low temperatures is limited, among other factors, by its inability to activate key Calvin cycle enzymes in the short term (Fig. 2) or to maintain their activation state in the long-term exposure to 10°C (Table II). The capacity for sucrose synthesis, whose reduced flux at low temperature can limit the rate of CO<sub>2</sub> fixation, would also suffer from a failure to increase the activity of SPS or its activation state (Table II). Sassenrath *et al.* (26) showed that, in the chilling-sensitive tomato plant, there was an accumulation of Fru 1,6-P<sub>2</sub> and Sed 1,7-P<sub>2</sub> after exposure to high light at low temperature. There was also a depletion of RuBP, indicating a restriction within the regenerative phase of the Calvin cycle relative to carboxylation. They showed that the light activation of stromal Fru 1,6-P<sub>2</sub>ase was restricted by prechilling tomato plants, suggesting that chilling disrupts the normal thioredoxin-dependent activation of the biphosphatases. In contrast, in bean, the activation state of NADP-MDH increased from 41 to 89% after 2 min at 10°C (Fig. 2), showing that no loss of the capacity for reductive activation occurred. However, Sassenrath *et al.* (26) did their experiments after a period of prechilling in the light, which may have inactivated the thioredoxin system to a degree not observed in these experiments.

The inability of bean to activate enzymes of the Calvin cycle, and hence to compensate for the lower temperature and to raise photosynthetic rates, is a feature that could contribute to photoinhibition and chilling sensitivity right from the onset of exposure to low temperature. However, the syndrome certainly involves more than a diminished capacity to activate such enzymes. The Chl fluorescence data for bean not only show reduced photochemical quenching at 10°C, but also only a very slow increase in nonphotochemical quenching (Fig. 4). Nonphotochemical quenching will reflect changes in energy-dependent quenching, and the lack of an increase suggests that energy-dissipating mechanisms are not coming into play in bean in the short term, which would increase its susceptibility to photoinhibition during prolonged periods at low temperature.

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